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MINIREVIEW

In Vitro Simulation of In Vivo Conditions: Physical State of the Culture Medium

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INTRODUCTION

In vitro testing may be useful in predicting how microbiological phenomena manifest themselves in living organisms, particularly in humans. The in vitro conditions of an experiment should attempt to replicate in vivo conditions as closely as possible. It is the purpose of this minireview to point out the influence of the physical state of a culture medium on in vitro experimental techniques that are designed to predict in vivo phenomena.

The ultrastructure, biochemical and antigenic characteristics, and antibiotic susceptibilities of bacteria are usually investigated by growing the organisms in a nutritionally balanced broth. Studies of microbial population dynamics and cell cultivation techniques use liquid media (1, 32). Automated procedures for species identification and antibiotic susceptibility testing also use liquid media (38).

Bacterial infection is preceded by bacterial colonization. Once adherence has taken place, the pathogen is able to establish local infection and subsequent invasion of a host (34, 35). Adherence of bacteria to a surface is usually a prerequisite for a pathogenic effect. There is substantial evidence that many infections result from organisms that adhere to surfaces as organized biofilms (6, 12). Most bacteria do not produce pathogenic effects in body fluids; they are found in body fluids as a result of shedding from the actual infection site, contamination from instrumentation, or rupture of a tissue that forms a natural physical barrier (24). The devastating effects of anthrax bacilli are not related to multiplication in blood, as once believed. Instead, clogging of capillaries with anthrax bacilli is due to multiplication in the lymph nodes with subsequent seeding of the bloodstream (42).

The in vivo multiplication rate of bacteria differs from the in vitro rate in conventional media. During an infection, bacterial generation time increases progressively and may be as long as 20 h, as in *Staphylococcus aureus* osteomyelitis in rabbits (45). The difference between in vivo and in vitro multiplication rates is due partly to in vivo nutrient depletion, such as the iron restriction seen in certain infections (3, 12, 41).

The first round of host defense against bacterial invasion is phagocytosis, and polymorphonuclear cells require a surface to deploy their activity (44). It appears, therefore, that in vitro systems that provide a solid support surface for the growth of bacteria should reflect in vivo conditions more accurately than do liquid media.

GROWTH IN A LIQUID VERSUS GROWTH ON A SOLID MEDIUM

Some effects related to the physical state of a culture medium have been observed with protoplasts. These organisms do not multiply in broth, grow into L-forms on soft agar, and revert to cell wall formation on hard agar; the harder the agar, the higher the reversion rate (17). When *Escherichia coli* is grown in liquid media, many cells assume an abnormal morphology as temperature decreases, but very few abnormal cells form on a solid medium (15). Strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis produce mucoid colonies on agar but turbid, nonmucoid, homogeneous suspensions of cells in broth (4). When certain strains of *Proteus mirabilis* are grown in liquid media, tumbling and chemotaxis are repressed and flagellum synthesis and insertion are derepressed (8). Growth of *Neisseria gonorrhoeae* can be inhibited by a substance isolated from *Streptococcus* (*Enterococcus*) *faecalis* on a solid medium but not in a liquid medium (9).

In one study, cell wall protein was extracted from 15 *S. aureus* isolates grown in a chemically defined liquid medium and in the same medium with 1% agar added. After separation of the cell wall antigens by gel electrophoresis and Western blots (immunoblots), the antigens were probed with chicken antiserum. For each of the 15 strains grown on agar, high-molecular-weight (120,000- to 220,000-molecular-weight) bands were either enhanced or changed significantly compared with antigens of the same strains grown in liquid medium without agar. Passage studies from solid to liquid media and vice versa demonstrated the ability of these staphylococci to alter surface protein when grown alternately in liquid and on solid media (5).

Pneumococci normally produce alpha-hemolysis when grown on blood agar. When pneumococci were grown on horse blood agar and incubated anaerobically, 39% of strains produced intense beta-hemolysis (26). All strains of pneumococci produce a ring of beta-hemolysis surrounding the zone of inhibition of a penicillin disk if the pneumococci are incubated anaerobically (28). Beta-hemolysis is not produced when pneumococci are grown in broth with erythrocytes and antibiotics (20). Similarly, 47% of strains of staphylococci produced a ring of beta-hemolysis surrounding inhibition zones produced by penicillin, cephalothin, oxacillin, or cycloserine disks (18). When staphylococci are grown in a liquid containing antibiotics and erythrocytes, however, beta-hemolysis does not occur (20). The hemolytic activity of leptospires against erythrocytes of various animal species is detectable only on solid culture media (39).

Susceptibility to antibacterial agents of organisms grown on agar differs from that of organisms grown in liquid media

(10). Blood culture isolates of *P. aeruginosa* grown in liquid medium demonstrate considerably greater sensitivity to serum bactericidal activity than do strains grown on agar (7). Significant variations in MICs for *Haemophilus influenzae* were observed with chloramphenicol, sulfamethoxazole-trimethoprim, erythromycin, and cefoperazone when solid media were used instead of the same media in liquid form (2). Cephalothin plus sulfamethoxazole-trimethoprim exerts a synergistic effect against strains of *Staphylococcus epidermidis* on Mueller-Hinton agar, but no synergy is observed when Mueller-Hinton broth is used (16). The MICs of fleroxacin and norfloxacin against *P. aeruginosa* and *E. coli* in liquid media remain constant when the inoculum is increased from 10^3 to 10^6 CFU. A corresponding 1,000-fold increase in inoculum size, however, leads to a 16-fold increase in the MIC of each quinolone when the test is conducted on agar (14).

The ultrastructure of bacteria is also a function of the physical state of the growth medium. When *E. coli* cells are grown in tryptic soy broth, the bacilli are 2 to 3 μm long and 0.5 to 0.6 μm thick, with a rough surface. When the same organisms are grown on a membrane placed on tryptic soy agar, the organisms are approximately the same length as with broth incubation but thinner and smooth surfaced (23). An *S. aureus* strain grown for 18 h in tryptic soy broth shows markedly thinner cell walls and cross walls than does the same strain grown on a membrane placed on tryptic soy agar (23). Another interesting illustration of changes in ultrastructure involves staphylococci exposed to penicillin on a hard surface, such as a filter membrane placed on agar. After several hours, the cells assume a large diameter of 2 to 4 μm and a mass 12 times that of a normal staphylococcus (19, 22, 27). The cells also contain multiple thick cross walls that hold together as many as 16 organisms. In comparison, staphylococci grown in a liquid medium, tryptic soy broth, containing the same concentration of penicillin become only slightly larger than normal (1.5 to 2 μm in diameter) and display very few cross walls (25, 30). When staphylococci are incubated in broth containing nafacillin (40), penicillin (33), or cephalothin (31), the cells are only slightly enlarged and show minor cell wall alterations. When the large staphylococci are transferred to drug-free agar, the cross walls lyse, liberating cells of normal size and growth rate (19).

BACTERIA IN VIVO

The study of bacterial structure in experimental staphylococcal osteomyelitis in rats shows organisms with thick cell walls that are comparable structurally to staphylococci grown in vitro on membranes (30). The smooth and relatively thin *E. coli* bacilli observed in experimental peritonitis in mice are also comparable to *E. coli* bacilli grown on a membrane (23). The most interesting finding is, however, that the large cells with multiple cross walls seen in staphylococci grown in vitro on filter membranes can be isolated from animals with experimental infections and from patients with staphylococcal infections treated with beta-lactam antibiotics. Mice with peritonitis induced experimentally with *S. aureus* Smith received oxacillin. Subsequent examination of the peritoneal fluid showed phagocytized staphylococci of normal size and free staphylococci that were 1.8 to 2.5 μm in diameter with multiple thick cross walls (25). When the experiment was repeated by using pretreatment with cyclophosphamide to suppress phagocytosis, the organisms were observed to be up to 12 times the normal size with multiple thick cross walls (30). Similar morphological changes have been identified in organisms isolated from rabbits with staphylococcal endocarditis treated with cloxacillin (29) and from patients with *S. epidermidis* endocarditis, pleural effusions containing staphylococci, and lower respiratory tract infections caused by gram-positive cocci, all of whom were treated with beta-lactam antibiotics (11, 25). Comparison of the structures of the staphylococci observed in the examples cited above shows that the ultrastructures of the organisms were virtually indistinguishable from the ultrastructures of staphylococci grown on a filter membrane placed on agar containing a beta-lactam antibiotic. This similarity supports the conclusion that selection of a solid-phase support, such as a filter membrane, for in vitro studies should be considered if such studies are to reflect bacterial ultrastructure in vivo (21, 25).

MECHANISMS OF ADAPTATION ACCORDING TO THE PHYSICAL STATE OF THE MEDIUM

Bacteria maintain homeostasis during environmental fluctuations through modulation of virtually every aspect of biological function. This can include alterations in metabolism, modification of gene expression, and chemotaxis for movement toward more favorable conditions (37). Receptor proteins in bacterial membranes which detect chemicals in the surrounding medium initiate biochemical transduction, which results in induction or repression of specific operons (13, 37).

In view of the adaptive abilities of bacteria, how can these organisms differentiate a hard surface from a liquid environment? What chemical differences are perceived by the bacteria when changing from growth on a surface to growth in a liquid medium and vice versa? The density of bacteria and the concentration of accumulated metabolites are two factors that are involved. For example, after about 12 h of growth at 36°C, a staphylococcus reaches about 10^7 CFU/ml in tryptic soy broth or generates a colony containing about the same number of organisms on tryptic soy agar (43). Since each organism has a mass of approximately 1.5 pg (22), the total mass of organisms becomes approximately 15 mg (wet weight) in 1 ml of broth or in one colony. In broth, this mass of bacteria is dispersed in a volume almost 70 times larger than its own. Furthermore, the released metabolites are continuously washed off during incubation. In contrast, a colony of bacteria grown on agar releases its metabolites in space-limited surroundings (32). Because the metabolites must diffuse through semisolid matter, drainage is slow and a high metabolite concentration engulfs the bacteria. Colony formation on agar or on tissue in vivo brings the organisms close together, enabling them to influence the local environment to a much greater extent than in a liquid medium (36). Factors such as nutrient and water availability, osmotic pressure, and rate of gas diffusion in a broth culture are quite different from those of a colony grown on agar (32). The beta-hemolysis produced by pneumococci on agar is probably the result of a high concentration of a metabolite in the space surrounding the colony. The same mass of bacteria dispersed in broth cannot produce the concentration of the metabolite required for hemolysis. Accumulation of metabolites acts as a repressor or an activator of a regulatory protein which, in turn, can either activate or inactivate a series of genes.

CONCLUSIONS

A large body of evidence shows that bacteria grown on a surface are significantly different from bacteria grown in

liquid medium. The differences include growth rate, adherence, and susceptibility to antibodies and antibacterial agents, as well as differences in the biochemical constitutions of the bacteria themselves and their metabolites. One major difference is in ultrastructure. There is strong evidence indicating that bacteria in vivo grow and produce disease on surfaces and not in body fluids. The identical ultrastructures of bacteria found in vivo and organisms grown in vitro on a surface support the conclusion that in vitro experiments aimed at duplicating in vivo conditions must be done on solid media. Interestingly, the commonly accepted phenotypic traits of staphylococci and other species are those observed in organisms grown in broth, although the ultrastructures of organisms grown in broth differ from those of organisms from an infected host. Characteristics of organisms grown in broth might, surprisingly, be artifactual.

In vitro systems are unable to reproduce precisely the complexity of in vivo conditions. This review documents the inadequacy of our attempt at reproducing these conditions. Despite this problem and the long list of differences between bacterial growth in vivo and in vitro that have been outlined, there are also many comparable characteristics which indicate that in vitro tests as performed in a clinical microbiology laboratory are helpful in establishing clinical diagnoses and predicting susceptibility to antibiotics.

A shift from liquid to solid media could, however, substantially reduce a major difference between in vitro and in vivo environments, further increasing the capability of reproducing in vivo phenomena in vitro.

Our efforts must be concentrated on achieving a greater understanding of what our work means while expanding research to approach the dynamics of the host-parasite relationship.

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